"Interim Progress Report for CDFA Agreement Number 15-0578-SA"
Project Title: Field testing transgenic grapevine rootstocks expressing CAP and PGIP proteins.

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Introduction

This proposal is a continuation to test chimeric anti-microbial protein (CAP) and polygalacturonase inhibitory protein (PGIP) as a means to clear and block the movement of *Xylella fastidiosa* and provide resistance to Pierce's disease (PD). Rootstocks (Thompson Seedless, TS) expressing these proteins individually are currently being evaluated in the field, this part of the study will be concluded this year. TS rootstock lines expressing either CAP or PGIP are showing promise in protecting against PD which is being validated with in-field inoculations. Since TS is not a rootstock these genes must be tested in a commercially relevant rootstock. A project was supported (11-0240-SA; 2011-2013) to accomplish this and was successful in expressing CAP in two commercially relevant rootstocks 101-14 (Christensen, 2003) and 1103. These transgenic CAP-expressing rootstocks will be tested in the greenhouse and field starting in 2016. Additionally, to address the concern that the protein components of the present CAP have a non-plant origin two additional CAP constructs were built using grapevine components (project 12-130-SA; 2012-2014). These are will be tested via expression in 101-14 and 1103 rootstocks and will be ready for greenhouse and field testing 2017 onward. The field introduction of these rootstocks is aimed at evaluating different lines to identify those with good efficacy in protecting grafted, sensitive scion cultivar Chardonnay from developing PD.

List of objectives

Objective 1. Complete the efficacy of current round of *in planta* expressed chimeric NE-CB and PGIP proteins to inhibit and clear *Xf* infection *in* xylem tissue and through the graft union in grapevines grown under field conditions.

Activity 1. Complete and conclude testing of the current round of plants in the field
Activity 2. Conduct greenhouse and field evaluation of CAP expressing 110-14 and 1103 rootstocks.

Description of activities conducted to accomplish each objective, and summary of accomplishments and results for each objective

Objective 1. Complete the efficacy of current round of *in planta* expressed chimeric NE-CB and PGIP proteins to inhibit and clear *Xf* infection *in* xylem tissue and through the graft union in grapevines grown under field conditions.

This objective has two activities; the first will conclude field testing of transgenic TS expressing either CAP or PGIP and identify the two best lines. The second will begin greenhouse followed by field testing of transgenic rootstocks in a commercially relevant background to identify lines that show resistance to PD.

Activity 1. Complete and conclude testing of the current round of plants in the field.
At the Solano County site (Fig. 1), half of the un-grafted transgenic lines were manually inoculated as described (Almeida et al. 2003) on July 13, 2011 and half on May 29, 2012. Half of the grafted transgenic lines were manually inoculated on the latter date. Ungrafted and grafted grapevines at the Solano site that were not previously inoculated were manually inoculated on June 17, 2013, completing the inoculations of all grapevines at this location. On May 27, 2014 and on May 27, 2015, following the recommendation of the Product Development Committee (PDC) of the Pierce's Disease Control Program, at least four new canes per year from all grafted transgenic and control plants at this site were mechanically inoculated with *Xf*. Inoculation dates from 2011 to 2015 are shown in a color-coded map (Table 1).

Figure 1. Solano County grafted transgenic grapevines inoculated in spring 2014 and spring 2015. Summer 2016

Table 1. Solano County grape field map, color-coded by *Xf* inoculation date, from 2012 to 2015.

	Vine 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Row 9	40-41-G	40-41-G	TS-50-G	TS-50-G	TS-50-G	40-92-G	40-92-G	40-92-G	40-89-G	40-89-G	40-89-G	TS-G	TS-G	TS-G	45-77-G	45-77-G	45-77-G	41-151-G	41-151-G	41-151-G	TS-G	TS-G	TS-G	TS-G	
Row 8	52-08-G	52-08-G	52-08-G	31-25-G	31-25-G	31-25-G	40-41-G	40-41-G	40-41-G	TS-50-G	TS-50-G	TS-50-G	40-89-G	40-89-G	40-89-G	TS-G	TS-G	TS-G	52-08-G	52-08-G	52-08-G	31-25-G	31-25-G	31-25-G	40-41-G
Row 7	31-25-G	40-92-G	40-92-G	40-92-G	TS-50-G	TS-50-G	TS-50-G	40-89-G	40-89-G	40-89-G	TS-G	TS-G	TS-G	45-77-G	45-77-G	45-77-G	40-92-G	40-92-G	40-92-G	41-151-G	41-151-G	41-151-G	45-77-G	45-77-G	45-77-G
Row 6	TS-50-G	TS-50-G	41-151-G	41-151-G	41-151-G	TS-G	TS-G	TS-G	40-92-G	40-92-G	40-92-G	40-89-G	40-89-G	40-89-G	41-151-G	41-151-G	41-151-G	52-08-G	52-08-G	52-08-G	40-41-G	40-41-G	40-41-G	31-25-G	31-25-G
Row 5													52-08-G	52-08-G	52-08-G	40-41-G	40-41-G	40-41-G	31-25-G	31-25-G	31-25-G	45-77-G	45-77-G	45-77-G	TS-50-G

North

Grapevine inoculatation with Xf (Temecula:Stag's leap mix, 60:40) at 250,000 per 20ul on 5/29/2012.
Grapevine inoculatation with Xf (Temecula) 250,000 per 20ul on 6/17/2013.
Grapevine inoculatation with Xf (Temecula:Stag's leap mix, 60:40) at 500,000 per 20ul on 5/27/2014.
Grapevine inoculatation with Xf (Temecula) at 500,000 per 20ul on 5/27/2015.

On July 22, 2014 and on September 15, 2015 one 2014-inoculated cane per grafted transgenic plant was harvested for quantification of *Xf* by qPCR using an Applied Biosystems SYBR Green fluorescence detection system. *Xf* DNA was extracted using a modified CTAB (hexadecyltrimethyl-ammonium-bromide) method that allowed us to obtain DNA with quantity and quality suitable for qPCR. The *Xf* 16s

primer pair (Forward 5'-AATAAATCATAAAAAAATCGCCAACATAAACCCA-3' and (Reverse 5'-AATAAATCATAACCAGGCGTCCTCACAAGTTAC-3') was used for *Xf* quantification. qPCR standard curves were obtained using concentrations of *Xf* ranging from 10^2 to 10^6 cells per 0.1 gm of tissue. *Xf* was detected in grafted transgenic vines, but with *Xf* counts that were lower than in grafted control grapevines.

Grapevine survival of grafted transgenic grapevines, inoculated in 2014/2015 was assessed on June 14th 2016 using a 1 to 5 score, where 1 = very healthy and vigorous grapevine; 2 = healthy grapevine and slightly reduced vigor; 3 = slightly reduced spring growth; 4 = much reduced spring growth and 5 = dead grapevine (Fig. 2). Grapevine survival rate was higher in most of the grafted inoculated transgenic lines from both strategies than in grafted untransformed control, with the CAP lines showing more efficiency in protecting grafted transgenic grapevines from developing PD.

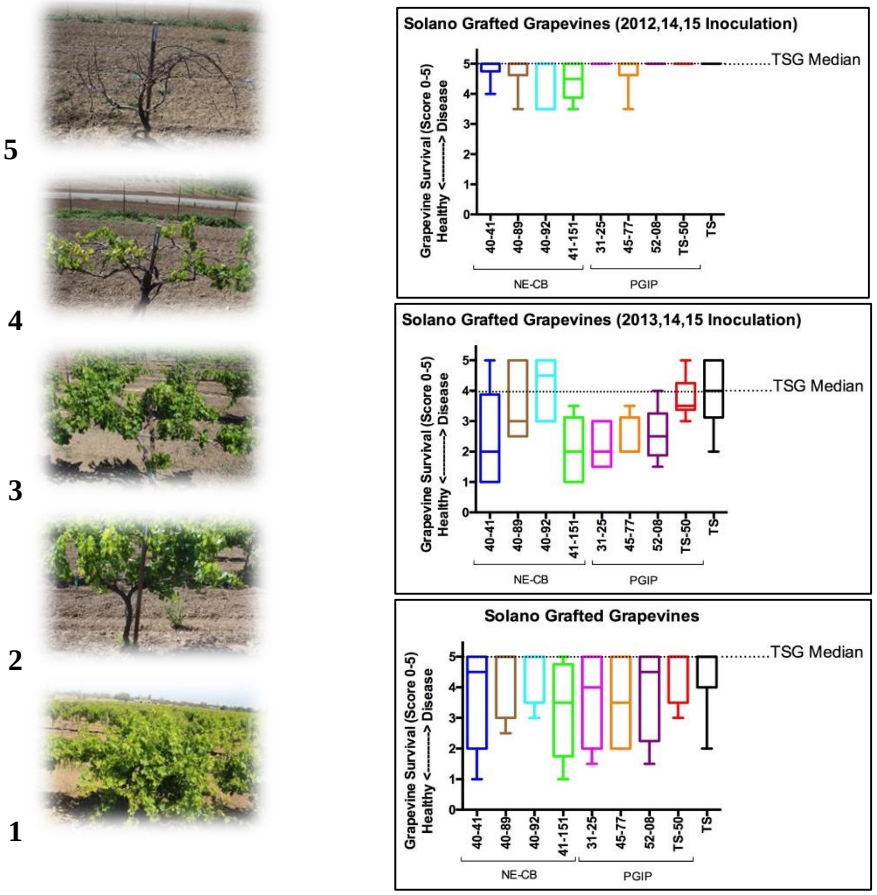

Figure 2. Grapevine survival of Solano grafted transgenic grapevine inoculated in 2012/2014-15 (upper right), 2013-2015 (middle right) and all inoculated grafted transgenic grapevines (lowe54r right, scored on June 14, 2016 using a scale of 1 to 5 (left).

Severity or absence of PD symptoms for all Solano County grafted transgenic grapevines inoculated from 2012 to 2015 was assessed on 2015 fall season using the PD disease symptom severity rating system 0-5, where 0 = healthy vine, all leaves green with no scorching; 1= first symptoms of disease, light leaf scorching on one or two leaves; 2 = about half the leaves on the cane show scorching; 3 = the majority of the of the cane shows scorching; 4 = the whole cane is sick and is declining and 5 = the cane is dead (Fig. 3). PD disease symptoms severity score was lower in most of the grafted inoculated transgenic lines from each strategy than in grafted untransformed control.

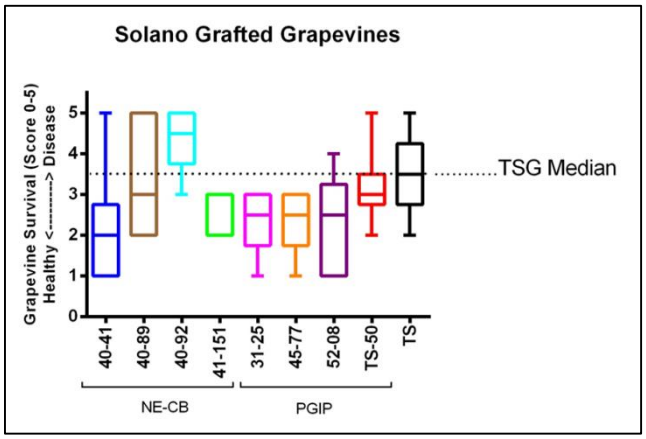

Figure 3. Severity or absence of PD symptoms for all Solano grafted inoculated grapevines scored on September 29th, 2015.

Activity 2. Conduct greenhouse and field evaluation of CAP expressing 101-14 and 1103 rootstocks. In this activity the focus is on green house and field testing of 6 vector constructs that are in the plant transfromation pipeling for the two commercially relevant rootstock 101-14 (101-14, Christensen, 2003) and 1103. The first two constructs shown in Figure 4 below were developed in a previously supported project (11-0240-SA; 2011-2013) with plants that are being propagated for testing described in detail below. The other four vector constructs were designed in a more recent project (project 12-130-SA; 2012-2014) that represent four additional CAP constructs were built using grapevine components. These are still in the transformation pipeline with the recovery ongoing for selection of 101-14 and 1103 transgenic CAP expressing rootstocks and will be ready for greenhouse and field testing 2017 onward.

Figure 4. CAP vectors used to create transgenic 101-14 MGT and 1103-P rootstocks that will be tested in greenhouse and field

In the case of the above two constructs the transformation is complete and there are currently 30 101-14 and seven 1103 transgenic lines for CAP-1 currently in the greenhouse being propagated and tested for resistance to PD. Approximately 20 transgenic each for 101-14 and 1103 for the CAP-2 construct are in the final tissue culture stage in the transformation pipeline and will be entering the greenhouse in early 2016. Since the yield for 1103 transformed with CAP-1 were low and new transformation was initiated in Aug 2015. A propagation/testing pipeline has been successfully developed for both 101-14 and 1103-P grapevines and the transgenic lines will be tested for PD resistance in the greenhouse as they emerge from the transformation.

The testing of the 101-14 and 1103 transformed rootstocks transformed with CAP-1 has already been initiated in the greenhouse and field testing of the resistant grapevine rootstocks will be initiated in fall of 2016. The field introduction of these rootstocks is aimed at evaluating their efficacy in protecting grafted sensitive Chardonnay grapevine variety from developing PD.

The 101-14 and 1103 transgenic rootstocks lines were screened for the presence of CAP-1 transgene using PCR. Those 101-14 and 1103 plants that that were PCR positive were clonally propagated for greenhouse testing. The clones were trained into a two-cane system and inoculated with *Xylella fastidiosa*. Plants were inoculated with 20uL of *Xf*. at a site roughly three nodes above the fork in the canes and eight leaves below the top of the cane, then it was turned over and inoculated with another 20uL of *Xf* directly behind the first inoculation point on the same cane for an inoculum of 8.72 x 10^6 *Xf* cells.

The transgenic rootstocks were evaluated for Pierce's disease symptoms for the first time 12 weeks post inoculation when the first disease symptoms became present, and every two weeks thereafter until a final score was taken 18 weeks post inoculation. A scoring system of 1-5 was used and a value was assigned according to the following: 1 = No visible disease symptoms (Good); 2 = Disease symptoms on less than 4 leaves (Good/OK), 3 = Disease symptoms exhibited on 50 percent the cane (4 leaves, OK); 4 = Disease symptoms exhibited on 75 percent of the cane (6 leaves, OK/Bad) and 5 = Symptoms stretching the entire length of inoculate cane (8 leaves, Bad).

One elite line of 101-14 presented no PD symptoms and got a score of 1. Five 101-14 plant lines got a score of 2, which look very promising and were considerably less sick than the untransformed 101-14 control which was scored a 5 (Fig 5). The six transgenic lines that scored a 1 or a 2 have been clonally propagated from the uninfected mother plants and are going to be screened in the field trial. All lines of 1103 scored bad and received a score of 5.

Figure 5: Infected WT 101-14 grapevines with disease symptoms running the entire length of the infected cane (top). The elite transgenic line that showed no symptoms 18 weeks post inoculation (bottom).

<u>Publications produced and pending, and presentations made that related to the funded project.</u>

Dandekar, A.M., D. Gilchrist, P. Rolshausen, A.M. Ibanez, A. Jacobson D. Dolan, R. Just and H. Gouran. 2015. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines filed trial. Research Progress Reports: Pierce's Disease and Other Designated Pests and Diseases of Winegrapes. December 2015. pp. 18-26.

Dandekar, A.M. D. Gilchrist, T. Miller, A.M. Ibanez, D. Dolan and H. Gouran. 2014. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines filed trial. Proceedings of Pierce's Disease Research Symposium held December 15-17, 2014 at the Sheraton Grand Sacramento Hotel, Sacramento, California. pp. 106-117.

Dandekar, A.M. D. Gilchrist, T. Miller, A.M. Ibanez, D. Dolan and H. Gouran. 2013. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines filed trial. Proceedings of Pierce's Disease Research Symposium held December 16-18, 2013 at the Hiatt Regency Hotel, Sacramento, California. pp. 95-100.

Dandekar, A.M., H. Gouran, A.M. Ibáñez, S.L. Uratsu, C.B. Aguero, S. McFarland, Y. Borhani, P.A. Feldstein, G. Bruening, R. Nascimento, L.R. Goulart, P.E. Pardington, A. Chaudhary, M. Norvell, E. Civerelo and G. Gupta. 2012. An engineered innate defense protects grapevines from Pierce's disease. Proc. Nat. Acad. Sci. USA 109: 3721-3725.

Dandekar, A.M., A.M. Ibáñez, D. Dolan, H. Gouran, D. Gilchrist and T. Miller. 2012. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines field trial. Proceedings of the Pierce's Disease Research Symposium, Dec 2012, pp. 94-103.

Dandekar, A.M., A.M. Ibáñez, H. Gouran, S. Uratsu, D. Gilchrist and T. Miller. 2011. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines field trial. Proceedings of the Pierce's Disease Research Symposium, Dec 2011, pp. 101-106.

Dandekar, A.M., A.M. Ibáñez, H. Gouran, S. Uratsu. D. Gilchrist and T. Miller. 2010. Chimeric antimicrobial protein and polygalacturonase-inhibiting protein transgenic grapevines field trial. Proceedings of the Pierce's Disease Research Symposium, Dec 2010, pp. 161-164.

Research relevance statement, indicating how this research will contribute toward finding solutions to Pierce's disease in California.

This proposal is a continuation of a project to test expression of a chimeric anti-microbial protein (CAP) and polygalacturonase inhibitory protein (PGIP) as a means to clear and block the movement of *Xylella fastidiosa* and provide resistance to Pierce's disease (PD). Rootstocks (Thompson Seedless, TS) expressing these proteins individually are currently being evaluated in the field, this study will build on this important research. TS rootstock lines expressing either CAP or PGIP are showing promise in protecting against PD which is being validated with in-field inoculations. Since TS is not a rootstock these genes must be tested in a commercially relevant rootstock which is what will be accomplished in this research. This research will test transgenic rootstocks developed in two previously funded projects (11-0240-SA; 2011-2013 and project 12-130-SA; 2012-2014) for providing trans-graft protection against PD. The greenhouse and field testing of these rootstocks is aimed at evaluating different lines to identify those with good efficacy in protecting grafted, sensitive scion cultivar Chardonnay from developing PD. Elite rootstock lines will be good candidates for commercialization.

Layperson summary of project accomplishments.

This research is a continuation of the field evaluation of CAP and PGIP expressing rootstocks to enable trans-graft protection of scion varieties of grapevine from developing PD. The research has two activities the first is to conclude the field testing of TS as a rootstock. Grapevine survival of grafted transgenic

grapevines, inoculated in 2014/2015 was assessed on June 14th 2016, data showed that survival rate for most grafted inoculated transgenic TS lines from both strategies is higher than in untransformed control, with the CAP lines showing more efficiency in protecting grafted transgenic grapevines from developing PD. Severity or absence of PD symptoms for all Solano County grafted transgenic grapevines inoculated from 2012 to 2015 was assessed in the 2015 fall season and PD disease symptoms severity score was lower in most of the grafted inoculated transgenic lines from each strategy than in grafted untransformed control.

The second activity of this project will focus on the field evaluation CAP constructs in commercially relevant rootstocks. We have initiated the evaluation of CAP-1 expressing 101-14 and 1103 rootstocks efficacy in protecting grafted sensitive Chardonnay grapevine variety from developing. The CAP-2 expressing 101-14 and 1103 are in the final tissue culture stage in the transformation pipeline and will be entering the greenhouse as they emerge from the transformation. The 101-14 and 1103 transgenic rootstocks lines were screened for the presence of CAP-1 transgene using PCR. Those 101-14 and 1103 plants that that were PCR positive were clonally propagated for greenhouse testing. Plants were trained into two cane system and one cane per plant was manually inoculated with 8.72×10^6 *Xf* cells. Inoculated transgenic rootstocks were evaluated for Pierce's disease symptoms 18 weeks post inoculation and six elites CAP-1 expressing 101-14 rootstock lines had very acceptable scores of 1 or 2. The six elite transgenic lines have been clonally propagated from the uninfected mother transgenic plants and are going to be screened in the field trial and field planting is expected in fall 2016.

Status of funds.

We have expended all the funds available for the period April 2016 to June 30, 2016.

Summary and status of intellectual property associated with the project.

An invention disclosure will be made for a plant patent once an elite transgenic rootstock line demonstrates excellent field efficacy in protecting a grafted sensitive scion from coming down with PD.

Literature cited.

Agüero, C.B., C.P. Meredith, and A.M. Dandekar. 2006. Genetic transformation of *Vitis vinifera* L. cvs. 'Thompson Seedless' and 'Chardonnay' with the pear PGIP and GFP encoding genes. Vitis 45:1-8.

Almeida, R.P.P., and A.H. Purcell. 2003. Biological traits of *Xylella fastidiosa* strains from grapes and almonds. App. Env. Microbiol. 68:7447-7452.

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